GATA4 and GATA6 Knockdown During Luteinization Inhibits Progesterone Production and Gonadotropin Responsiveness in the Corpus Luteum of Female Mice[1]

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ABSTRACT

The surge of luteinizing hormone triggers the genomic reprogramming, cell differentiation, and tissue remodeling of the ovulated follicle, leading to the formation of the corpus luteum. During this process, called luteinization, follicular granulosa cells begin expressing a new set of genes that allow the resulting luteal cells to survive in a vastly different hormonal environment and to produce the extremely high amounts of progesterone (P4) needed to sustain pregnancy. To better understand the molecular mechanisms involved in the regulation of luteal P4 production in vivo, the transcription factors GATA4 and GATA6 were knocked down in the corpus luteum by crossing mice carrying *Gata4* and *Gata6* floxed genes with mice carrying Cre recombinase fused to the progesterone receptor. This receptor is expressed exclusively in granulosa cells after the luteinizing hormone surge, leading to recombination of floxed genes during follicle luteinization. The findings demonstrated that GATA4 and GATA6 are essential for female fertility, whereas targeting either factor alone causes subfertility. When compared to control mice, serum P4 levels and luteal expression of key steroidogenic genes were significantly lower in conditional knockdown mice. The results also showed that GATA4 and GATA6 are required for the expression of the receptors for prolactin and luteinizing hormone, the main luteotropic hormones in mice. The findings demonstrate that GATA4 and GATA6 are crucial regulators of luteal steroidogenesis and are required for the normal response of luteal cells to luteotropins.

corpus luteum, female infertility, GATA, luteinizing hormone (LH/LH receptor), progesterone/progesterone receptor

INTRODUCTION

The corpus luteum is one of the most remarkable endocrine glands in the human body and, in proportion to its mass, is the most active steroid-secreting tissue. The formation of the corpus luteum is triggered by the midcycle surge of luteinizing hormone (LH), which stimulates the final maturation of the oocyte and its release at ovulation and converts the ovulated follicle into a new endocrine gland that produces almost exclusively progesterone (P4), a steroid hormone. Luteal P4 promotes uterine receptivity for blastocyst implantation. Despite the vital role of the corpus luteum in female fertility, the molecular mechanisms involved in the maintenance of luteal function and steroidogenesis are not fully understood.

Luteal cells from rodents, farm animals, and humans express the transcription factors GATA4 and GATA6 [1–5]. Insights into the role of GATA4 in luteal steroidogenesis are exclusively provided by in vitro transactivation experiments showing that this factor can target the promoter of steroidogenic genes, including steroidogenic acute regulatory protein (STAR) [2, 6–9], cholesterol side-chain cleavage (CYP11A1) [10], 3β-hydroxysteroid dehydrogenase (HSD3B1) [11], aromatase (CYP19A1) [9, 12–14], inhibin alpha (INHA) [2, 9, 15, 16], anti-Müllerian hormone [9], and others (for a review, see [17]). However, transactivation experiments do not necessarily recapitulate the in vivo regulation of luteal function. The only in vivo evidence suggesting the involvement of GATA on the regulation of luteal steroidogenesis is provided by the colocalization of GATA4/6 and CYP11A1 to the corpus luteum of rats [1]. Finally, in vitro experiments using small interference RNA (siRNA) in porcine luteal cells showed that GATA6 knockdown reduces *Star* mRNA and P4 levels by 60%, whereas knockdown of GATA4 has no effect on basal and cAMP-induced P4 production or *Star* expression [18]. These findings suggest that GATA6 plays a more dominant role on the regulation of luteal P4 production; nevertheless, functional in vivo studies are needed to support this conclusion and to determine the role of GATA4 and GATA6 on the regulation of luteal function.

Previously, we demonstrated that the conditional knockdown of GATA4 and GATA6 in granulosa cells disrupts follicle development and ovulation [19, 20]. This block in follicular growth prevents the formation of corpora lutea; therefore, studies to examine the roles of GATA4 and GATA6 in luteal function were not possible. Here, GATA4 and GATA6 functions in luteal cells and the molecular mechanisms involved in the regulation of luteal P4 synthesis in vivo were examined by targeting the *Gata4* and *Gata6* genes during luteinization using the Cre/lox system. This approach demonstrated that GATA4 and GATA6 are essential for luteal P4 synthesis and consequently for the maintenance of pregnancy in mice.

MATERIALS AND METHODS

Reagents

DMEM/F12 medium, equine chorionic gonadotropin (eCG), human chorionic gonadotropin (hCG), P4, and all other media components were purchased from Sigma unless otherwise specified.

Animals

Animals expressing Cre recombinase fused to the progesterone receptor (PRcre) were previously described [21]. *Gata4$^{f/f}$* mice were kindly provided by Dr. William Pu (Department of Cardiology, Children's Hospital Boston). *Gata6$^{f/f}$* mice were obtained from the Jackson Laboratory. The mice carrying floxed alleles of the *Gata4* and/or the *Gata6* genes were previously described [20]. PRcre and *Gata4$^{f/f}$*, *Gata6$^{f/f}$*, or *Gata4/6$^{f/f}$* mice were crossed to generate *PRcre:Gata4$^{f/f}$*, *PRcre:Gata6$^{f/f}$*, and *PRcre:Gata4/6$^{f/f}$* mice. Only *PR$^{+/cre}$* animals were used because *PR$^{cre/cre}$* animals are infertile [21]. The animals were treated in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals, and all the protocols were approved by the University of Illinois at Chicago Animal Care Committee.

Estrous Cycling

Vaginal smears were collected daily from adult females, that is, ~Day 55 (~d55), over the course of 14 days. The vaginal smears were evaluated microscopically to determine the stage of the cycle.

Ovulation Assay

Adult females (d90–d100), were injected subcutaneously with 7.5 international units (IU) of eCG for 48 h followed by treatment with 7.5 IU of hCG to induce ovulation. Next, 17 h after the hCG injection, the animals were euthanized and oocytes from each oviduct were collected and counted.

Immunohistochemistry

Immunohistochemistry was performed as previously described [20]. Slides were developed using a VECTASTAIN Elite ABC kit and 3,3′-diamidino-benzidine (Vector Laboratories) following the manufacturer's recommendations and then counterstained with Gill hematoxylin.

Western Blot Analysis

The control and *PRcre:Gata4/6$^{f/f}$* mice were treated with 7.5 IU of eCG followed by 7.5 IU of hCG for 96 h to induce ovulation and luteinization. Corpora lutea were isolated and then homogenized in ice-cold RIPA lysis buffer using a Dounce tissue grinder, followed by sonication. Samples were centrifuged to eliminate cellular debris, and the protein lysate was collected. Protein concentration was determined using the Pierce BCA Protein Kit Assay (Life Technologies) with bovine serum albumin as the standard. The samples were denatured using sample buffer followed by boiling at 90°C for 10 min. Twelve micrograms of protein were separated on 12% Bis-Tris-PAGE gels. Samples were transferred to nitrocellulose membranes followed by incubation in 5% nonfat dry milk for 2 h at room temperature to block nonspecific binding. Membranes were washed with 2 mM Tris, 15 mM NaCl, and 0.1% Tween 20, and incubated overnight at 4°C with anti-GATA4 (sc9053, 1:500 dilution; Santa Cruz), anti-GATA-6 (sc9055, 1:100 dilution; Santa Cruz), STAR (1:1000 dilution, kindly donated by Dr. D.B. Hales), and anti-β-actin (ACTB) (ab8227, 1:500 dilution; Abcam). Membranes were washed and incubated with an anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:10 000 dilution; Jackson ImmunoResearch) for 2 h at room temperature. Protein-antibody complexes were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) depending on the abundance of the protein and the sensitivity of the antibody.

RNA Isolation and Quantitative Real-Time PCR Analysis

The control, *PRcre:Gata4$^{f/f}$*, *PRcre:Gata6$^{f/f}$*, and *PRcre:Gata4/6$^{f/f}$* mice were treated with 7.5 IU of eCG followed by 7.5 IU of hCG for 36, 48, 72, or 96 h to induce ovulation and luteinization. Corpora lutea were isolated and then homogenized in 1 ml of TRIzol reagent (Invitrogen) using a Dounce tissue grinder. Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer's instructions. One microgram of total RNA was reverse transcribed at 42°C for 1 h using Moloney murine leukemia virus reverse transcriptase (Invitrogen). The cDNA was diluted to 100 μl in water, and 5 μl of this dilution was used for each reaction. The identity and size of all the PCR products were confirmed by sequencing and gel analysis. The results are expressed as the ratio between the copies per nanogram of total RNA of the gene of interest and ribosomal protein L19 (*Rpl19*). Primer sequences are provided in Table 1.

Progesterone Assessment

Truncal blood was collected from the control, *PRcre:Gata4$^{f/f}$*, *PRcre:Gata6$^{f/f}$*, or *PRcre:Gata4/6$^{f/f}$* mice under two conditions: 1) control, *PRcre:Gata4$^{f/f}$*, *PRcre:Gata6$^{f/f}$*, or *PRcre:Gata4/6$^{f/f}$* mice were injected with 7.5 IU of eCG for 48 h followed by treatment with 7.5 IU of hCG for 17, 24, 36, 48, 72 or 96 h to induce ovulation and luteinization or 2) control or *PRcre:Gata4/6$^{f/f}$* female mice were mated with males of proven fertility until confirmation of copulation by the presence of a vaginal plug. Truncal blood was collected from the mice at the time of harvest. Plasma was isolated by centrifugation of blood at $3000 \times g$ for 5 min. Progesterone plasma levels were determined using a DRG Progesterone ELISA kit. Progesterone levels were determined in the control plasma samples at a 1:20 dilution and in the *PRcre:Gata4$^{f/f}$*, *PRcre:Gata6$^{f/f}$*, and *PRcre:Gata4/6$^{f/f}$* plasma samples at a 1:5 dilution. The sensitivity of the P4 assays used was 0.045 ng/ml.

Luteal Cell Culture

Luteinizing granulosa cells were isolated from preovulatory follicles from immature 23-day-old *Gata4/6$^{f/f}$* mice treated with eCG for 48 h followed by hCG treatment for 7 h. Cells were infected with adenoviral Cre recombinase (AdCRE) (University of Iowa Gene Vector Transfer Core) or virus-expressing green fluorescent protein (GFP) at a multiplicity of infection of 10. For RNA expression, cells were treated with AdCRE or AdGFP for 48 h prior to treatments with 1 mM N6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate (dbcAMP) or 50 ng/ml hCG; dbcAMP is a cell-permeable cAMP analog that activates cAMP-dependent protein kinase (PKA). Cells were cultured for 48 h after treatments and then harvested for RNA isolation.

Statistics

Data are expressed as the mean ± SEM. Multiple group statistical analyses were performed by one-way ANOVA followed by the Tukey test for multiple comparisons. Two-group comparisons were performed using a *t*-test for independent samples. Differences between groups were considered to be statistically significant at $P < 0.05$.

RESULTS

Deletion of GATA4 and GATA6 During Luteinization Leads to Female Infertility

To examine the roles of GATA4 and GATA6 in corpus luteum function, mice bearing floxed *Gata4* and *Gata6* genes were crossed with PRcre mice. In PRcre mice, Cre recombinase is expressed only in granulosa cells during the luteinization process and leads to the targeted deletion of floxed genes in the corpus luteum [21]. Using this approach, *Gata4* and *Gata6* were targeted alone or together via the generation of *PRcre:Gata4$^{f/f}$*, *PRcre:Gata6$^{f/f}$*, and *PRcre:Gata4/6$^{f/f}$* mice, respectively. As controls, *PRcre:Gata4/6$^{f/+}$* mice were used. The control and *PRcre:Gata4/6$^{f/f}$* mice were treated sequentially with eCG and hCG as described in the *Materials and Methods* section. Immunohistochemical analysis of the ovaries of these mice revealed that GATA4 and GATA6 proteins were decreased in the corpus luteum 96 h after hCG treatment (Fig. 1A). Western blot analysis showed significantly diminished expression of GATA4 and GATA6 proteins in the corpus luteum of *PRcre:Gata4/6$^{f/f}$* mice when compared with the controls (Fig. 1B).

Next, we evaluated the main reproductive functions of the control and GATA conditional knockdown mice. No differences in the estrous cycle length or ovulation rate were detected between the control and *PRcre:Gata4$^{f/f}$*, *PRcre:Gata6$^{f/f}$*, or *PRcre:Gata4/6$^{f/f}$* female mice (Fig. 2, A and B, and data not shown). In addition, vaginal plugs were found in the control and experimental females after mating with wild-type males (data not shown).

The mating of the control and experimental animals with males of proven fertility for 6 mo demonstrated that *PRcre:Gata4$^{f/f}$* and *Prcre:Gata6$^{f/f}$* females are subfertile,

Gene	Forward primer (5′→3′)	Reverse primer (3′→5′)	Product size (bp)
Rpl19	CAATGAGACCAATGAAATCG	GCAGTACCCTTCCTCTTCC	297
Gata4	GAAGACACCCCAATCTCG	TTGTGATAGAGGCCACAGG	199
Gata6	AAACGCCGGTGCTCCACAGCTTACAGG	TGATGAAGGCACGCGCTTCTGTGGC	248
Star	TTTTGGGGAGATGCCGGAGC	GCGAACTCTATCTGGGTCTGCG	189
Cyp11a1	GATGTTCCACACCAGTGTCCC	AGGGTACTGGCTGAAGTCTCGC	178
Hsd3b1	CATTCTGGATGCCCAGTGCC	ATGAAGGCTGGCACACTTGC	177
Lhr	TGTAACACAGGCATCCGGACC	ACTCCAGCGAGATTAGCGTCG	220
Prlr	GAGAAAAACACCTATGAATGTC	GTAAGTCACATCCACATAAAGT	165
Inhbb	GTGAACCAGTACCGCATGCG	ACACTCCTCCACGATCATGTTGG	150

characterized by a significant decrease in the number of pups per litter when compared to the control mice (Fig. 2C). In marked contrast to the single knockouts, *PRcre:Gata4/6^{f/f}* females were infertile (Fig. 2C). This result agrees with serum

P4 levels determined 9 days after copulation, which showed that *PRcre:Gata4/6^{f/f}* females had significantly lower levels of P4 in circulation when compared with the control mice (Fig. 2D). These results indicate that the infertility of *PRcre:Gata4/6^{f/f}* females is not due to ovarian acyclicity, anovulation, or abnormal sexual behavior, but is most probably due to luteal dysfunction.
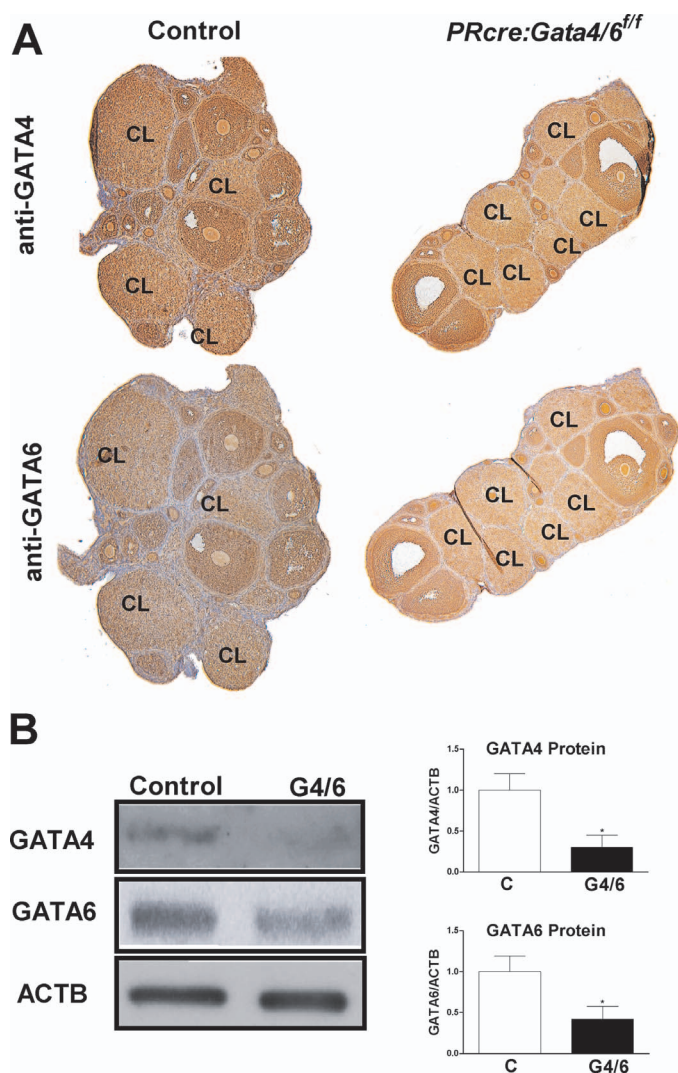
GATA Factors Are Required for Luteal P4 Production

Because only *PRcre:Gata4/6^{f/f}* mice are infertile and produce extremely low levels of P4, we next examined luteal function in the *PRcre:Gata4/6^{f/f}* mice, and P4 levels were measured in the serum of animals treated sequentially with eCG and hCG. As shown in Figure 3A, a time-course experiment revealed that P4 levels in the *PRcre:Gata4/6^{f/f}* mice were comparable to those found in the control animals at 17 and 24 h after hCG treatments. At 36 h, the P4 levels in the *PRcre:Gata4/6^{f/f}* mice tended to be lower than in the controls, although the difference was not statistically significant ($P = 0.08$). However, P4 levels were significantly lower in the *PRcre:Gata4/6^{f/f}* mice when compared with the controls at 48, 72, and 96 h after hCG treatment.

In the control mice, *Gata4* mRNA expression tended to decrease over time after hCG treatment (Fig. 3B, left). However, *Gata4* mRNA levels were not significantly different between the control and *PRcre:Gata4/6^{f/f}* mice 36 and 48 h after treatment with hCG. *Gata4* mRNA levels tended to be lower in the *PRcre:Gata4/6^{f/f}* mice than in the controls 72 h after hCG. A significant decrease in *Gata4* mRNA levels was observed in the *PRcre:Gata4/6^{f/f}* mice 96 h after hCG treatment when compared with the control animals.

In contrast to *Gata4,* the *Gata6* mRNA levels in the control mice increased significantly 48 and 72 h after hCG when compared to 36 h for the control group (Fig. 3B, right). These increases in luteal *Gata6* expression did not occur in the *PRcre:Gata4/6^{f/f}* mice. Thus, *Gata6* mRNA levels tended to be lower at 36 h and were significantly lower in the *PRcre:Gata4/6^{f/f}* mice after 48, 72, and 96 h when compared to the controls.

To determine the chronological development of P4 synthesis inhibition, the luteal mRNA content of *Star,* *Cyp11a1,* and *Hsd3b1,* three genes essential for P4 synthesis, was quantified 36, 48, 72, and 96 h following hCG treatment. In the control mice, *Star* mRNA levels increased from 36 to 48 h after hCG (Fig. 3C). However, the steady-state levels of *Star* mRNA were significantly decreased in the *PRcre:Gata4/6^{f/f}* mice at all times after hCG (Fig. 3C). The percentage of *Star* decrease in the *PRcre:Gata4/6^{f/f}* mice was only 50% at 36 h, whereas the inhibitions at 48, 72, and 96 h were 92%, 94%, and 96%, respectively. In contrast, no significant differences in *Cyp11a1* mRNA levels were observed between the control and *PRcre:Gata4/6^{f/f}* mice 36, 48, and 72 h after hCG. However, a significant decrease in *Cyp11a1* mRNA levels was observed 96

FIG. 1. Expression of GATA4 and GATA6 in *PRcre:Gata4/6^{f/f}* mice. Control (C) or *PRcre:Gata4/6^{f/f}* mice were treated with 7.5 IU of eCG for 48 h, followed by treatment with 7.5 IU of hCG for 96 h. **A**) Immunohistochemical analysis of GATA4 and GATA6 protein expression in whole ovary sections. CL: corpus luteum. **B**) Corpora lutea isolated from the control and *PRcre:Gata4/6^{f/f}* (G4/6) mice were used for the isolation of total protein for Western blot analysis to detect GATA4, GATA6, and β-actin (ACTB) (*$P < 0.05$ vs. C, *t*-test, n = 5).
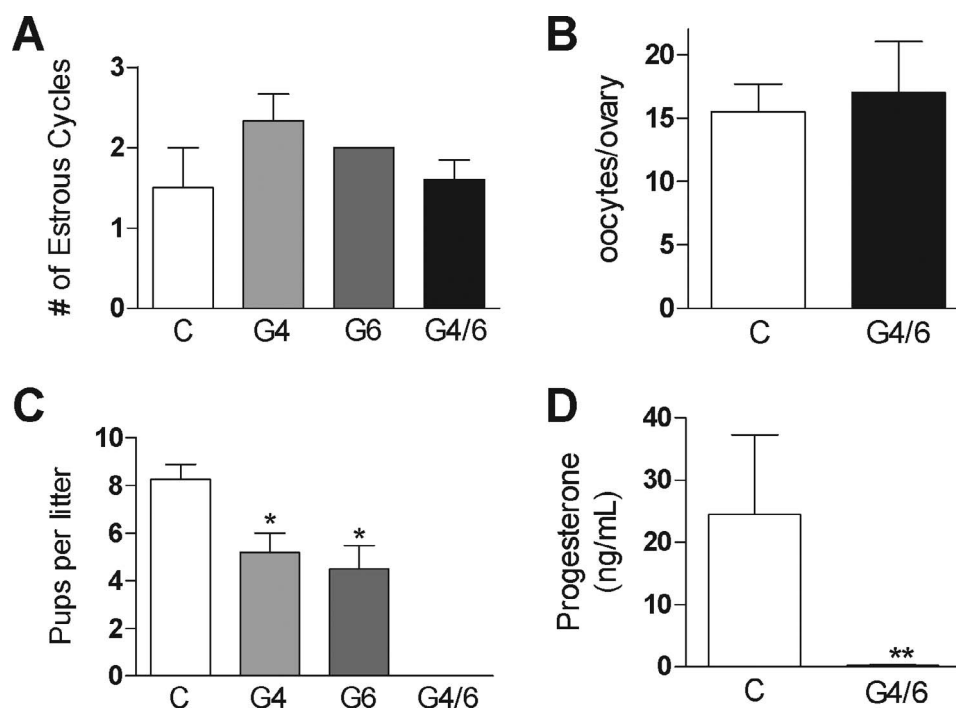
FIG. 2. Fertility, cyclicity, and ovulation of GATA-deficient mice. **A**) Number of estrous cycles over a 14-day period in *PRcre* (C), *PRcre:Gata4^f/f* (G4), *PRcre:Gata6^f/f* (G6), and *PRcre:Gata4/6^f/f* (G4/6) mice. **B**) Number of ovulated oocytes in control and G4/6 females 17 h following hCG treatment. **C**) Average number of pups per litter in C, G4, G6, and G4/6 female mice after continuous mating over a 6-mo period with males of proven fertility (*$P < 0.05$ vs. C, one-way ANOVA, n = 3). **D**) Serum P4 levels in female mice 9 days postcoitum (**$P < 0.01$ vs. control, *t*-test, n = 5).

h after hCG treatment in the *PRcre:Gata4/6^f/f* mice when compared with the controls.

As observed for *Gata6* and *Star* mRNA levels, *Hsd3b1* mRNA levels significantly increased from 36 to 48 h after hCG in the control animals. This increase did not take place in the *PRcre:Gata4/6^f/f* mice. Consequently, although the mRNA levels of *Hsd3b1* were not different at 36 h between the control and *PRcre:Gata4/6^f/f* mice, they were significantly lower in the *PRcre:Gata4/6^f/f* mice when compared with the controls at all later times (Fig. 3B).

The early inhibition of *Star* suggests that it may be a direct target of GATA factors in vivo; therefore, we performed Western blot analyses to examine whether the STAR protein is also affected. In the *PRcre:Gata4/6^f/f* mice, a 70% reduction in luteal STAR protein content was observed when compared to the controls (Fig. 3D).

Effect of P4 Replacement on Implantation in PRcre:Gata4/6^f/f Mice

Thus far, our results indicate that the recombination of *Gata4* and *Gata6* floxed genes during luteinization results in the inhibition of luteal P4 synthesis. To determine whether the lack of luteal P4 production is the primary cause of infertility, *PRcre:Gata4/6^f/f* female mice at d90–d100 were bred with males of proven fertility until copulation was confirmed by the presence of vaginal plugs. Starting the day after vaginal plugs were found, the females were injected daily with 3 mg of P4 or sesame oil for 13 days. On Day 14, implantation was assessed. The *PRcre:Gata4/6^f/f* mice injected with sesame oil alone showed no indication of implantation (n = 3). In contrast, in the *PRcre:Gata4/6^f/f* mice injected with P4, implantation sites were observed in three out of five mice (data not shown).

Role of GATA4 and GATA6 in the Expression of Luteotropic Hormone Receptors

It is well known that hCG strongly stimulates luteal P4 synthesis [22]. However, in the *PRcre:Gata4/6^f/f* mice, hCG was unable to stimulate serum P4 levels or the expression of *Star* and *Hsd3b1*. These results suggest that diminished expression of GATA4 and GATA6 may affect the response of luteal cells to LH. To test this possibility, the expression of the receptor for LH (LHR) at different times after the administration of hCG was examined. As shown in Figure 4, a significant decrease in *Lhr* mRNA levels was observed in the *PRcre:Gata4/6^f/f* mice when compared to the control animals at 48, 72, and 96 h after hCG. Moreover, in the control mice, *Lhr* mRNA levels increased progressively from 36 to 48 h after hCG treatment and remained high until 96 h after treatment. In contrast, in the *PRcre:Gata4/6^f/f* mice, a progressive decrease in *Lhr* expression from 36 to 96 h was observed (Fig. 4).

Because prolactin is essential for normal luteal function in rodents [22], prolactin receptor (*Prlr*) mRNA levels in the control and *PRcre:Gata4/6^f/f* mice were examined. In contrast to *Lhr*, the mRNA levels of *Prlr* were not affected 36 and 48 h after hCG. However, a significant decrease in the steady-state levels of *Prlr* mRNA was observed 72 and 96 h after hCG (Fig. 4).

GATA4 and GATA6 Contribution to Luteal Steroidogenesis and Lhr Expression

Next, we determined the individual contribution of GATA4 and GATA6 to the regulation of *Star*, *Cyp11a1*, *Hsd3b1*, and *Lhr* mRNA levels. For this purpose, the control, *PRcre:Gata4^f/f*, and *PRcre:Gata6^f/f* females were treated with eCG for 48 h, followed by treatment with hCG. Animals were killed 96 h after hCG administration. As shown in Figure 5,
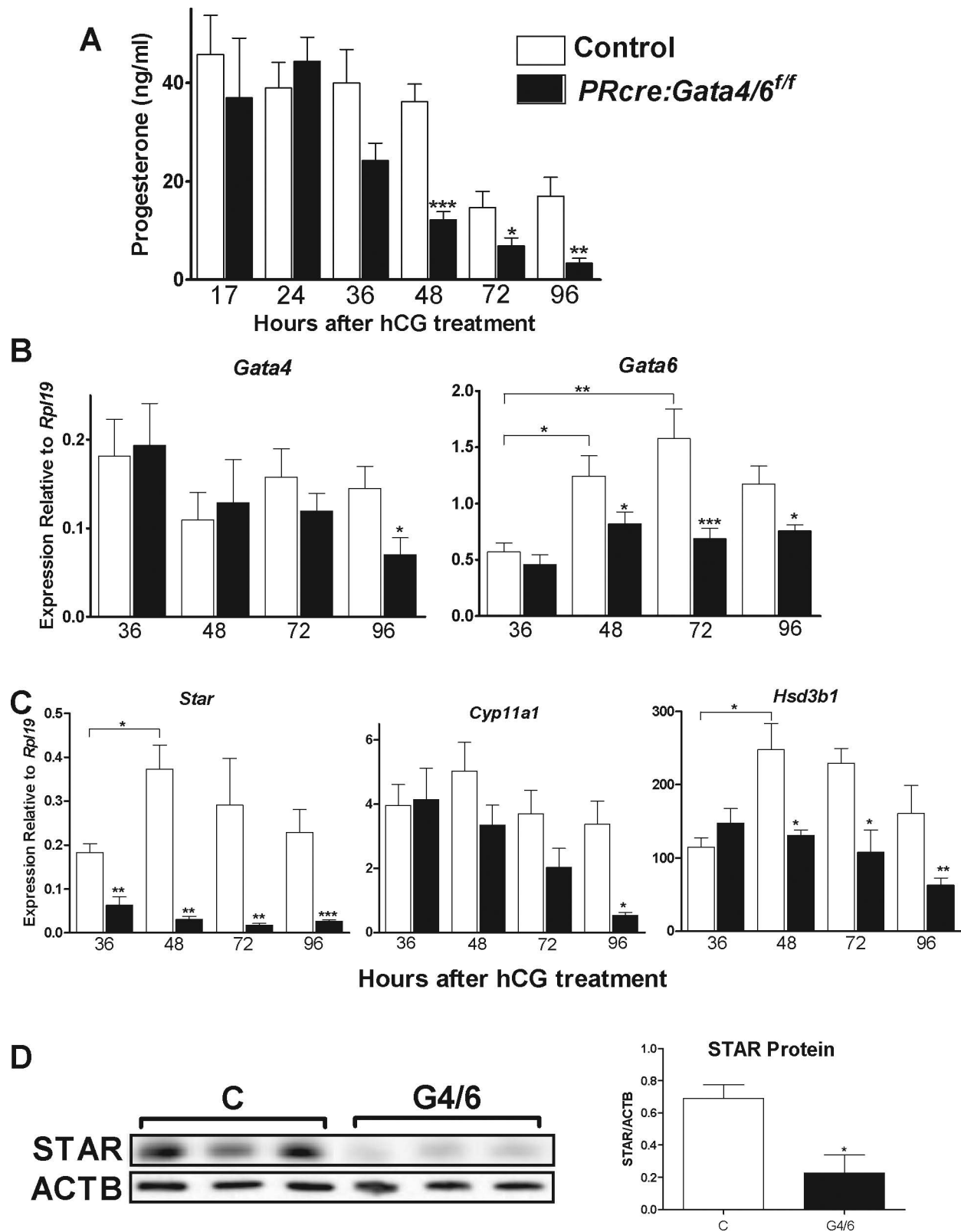
FIG. 3. Serum P4 and luteal steroidogenic gene expression in *PRcre:Gata4/6^{f/f}* mice. **A**) Serum P4 levels were measured in control (C) or *PRcre:Gata4/6^{f/f}* (G4/6) female mice treated with eCG followed by treatment with hCG for 17, 24, 36, 48, 72, or 96 h ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. C, one-way ANOVA, n = 5). **B** and **C**) *Gata4, Gata6, Star, Cyp11a1,* and *Hsd3b1* mRNA levels in C and G4/6 female mice treated with eCG and hCG for 36, 48, 72, or 96 h. Quantification of mRNA expression was performed using quantitative PCR ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. C or vs. 36 h as indicated by *t*-test, n = 8). **D**) Total protein isolated from the corpus luteum of C or G4/6 female mice treated with hCG for 96 h. Western blot analysis was used to detect STAR total protein levels. Quantification was performed as a ratio of STAR to ACTB as a loading control ($*P < 0.05$ vs. C, *t*-test, n = 5).
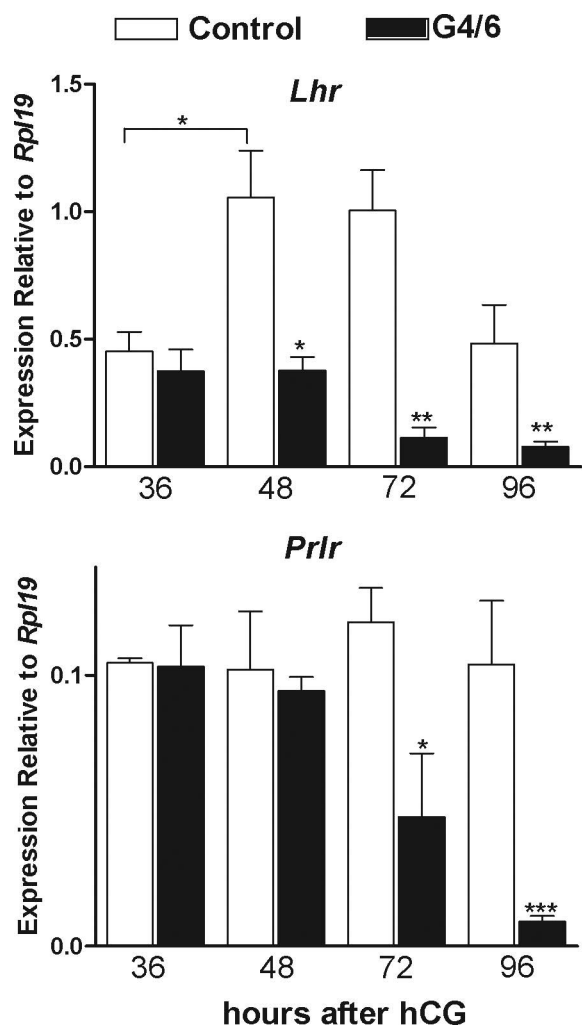
FIG. 4. Role of GATA4/6 knockdown on luteotropic receptor expression. Control (C) or *PRcre:Gata4/6^{f/f}* (G4/6) mice were treated with 7.5 IU of eCG for 48 h followed by treatment with 7.5 IU of hCG for 36, 48, 72, or 96 h. Total RNA was isolated from the corpus luteum of the C or G4/6 female mice treated with hCG for 96 h. Quantification of gene expression of *Lhr* and *Prlr* was performed using quantitative PCR (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. C, *t*-test, $n = 8$).

Lhr, *Star*, and *Hsd3b1* mRNA levels were significantly decreased in all the experimental animals when compared to the controls. In contrast, the mRNA of *Cyp11a1* was significantly decreased only in the *PRcre:Gata4^{f/f}* mice. *Cyp11a1* mRNA levels tended to be lower in the *PRcre:Gata6^{f/f}* mice; however, this decrease did not reach statistical significance. Additionally, serum P4 levels were significantly decreased in the *PRcre:Gata4^{f/f}* and *PRcre:Gata6^{f/f}* mice when compared to the control mice.

Direct and Indirect Effects of GATA on Luteal Steroidogenesis

Our findings reveal a decrease in the steady-state levels of the mRNAs for *Star*, *Cyp11a1*, *Hsd3b1*, *Prlr*, and *Lhr* in mice with reduced expression of GATA4 and GATA6 in the corpus luteum. However, because LH stimulates *Star*, *Cyp11a1*, and *Hsd3b1* expression [23], the downregulation of these genes could be due to a reduction in *Lhr* expression. To explore this possibility, we examined whether treatment with an analog of cAMP, the main second messenger of the LHR, restores *Star*,
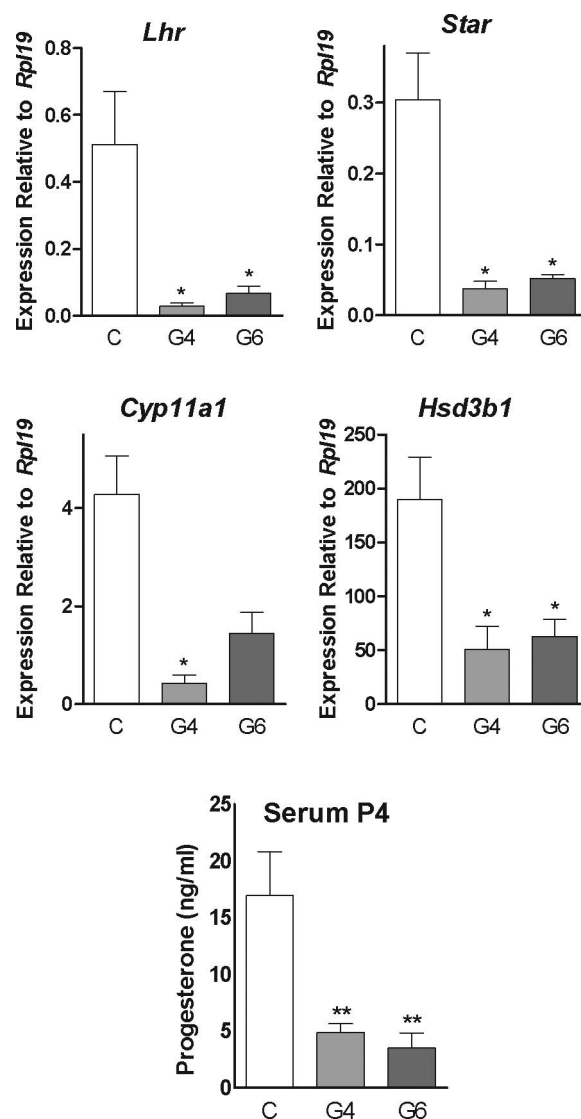

FIG. 5. Contribution of GATA4 and GATA6 to luteal steroidogenesis and *Lhr* expression. Control (C), *PRcre:Gata4^{f/f}* (G4), or *PRcre:Gata6^{f/f}* (G6) mice were treated with 7.5 IU of eCG for 48 h followed by treatment with 7.5 IU of hCG. **A**) Total RNA was isolated from the corpus luteum of the C, G4, or G6 female mice treated with hCG for 96 h. Quantification of gene expression of *Lhr*, *Star*, *Cyp11a1*, and *Hsd3b1* was performed using quantitative PCR (*$P < 0.05$ vs. C, $n = 8$, *t*-test). **B**) Serum P4 levels were measured in the C, G4, and G6 female mice treated with hCG for 96 h (**$P < 0.01$ vs. C, *t*-test, $n = 4$).

Cyp11a1, and *Hsd3b1* mRNA levels in GATA4/6-deficient cells. For this purpose, the *Gata4/6^{f/f}* immature female mice were treated with eCG for 48 h followed by hCG. Luteal cells were isolated 7 h after hCG administration at a time when luteinization had been initiated [24]. To ensure full recombination of the floxed genes in vitro, the cells were infected with AdCRE. As controls, cells were infected with a virus-expressing GFP. The overexpression of Cre reduced *Gata4* and *Gata6* mRNA to almost undetectable levels, whereas in cells infected with the control virus, *Gata4* and *Gata6* expression was not affected (Fig. 6A).

Next, we assessed the response of control and GATA4/6-deficient luteal cells to hCG or dbcAMP, an analog of cAMP. In the control cells, dbcAMP and hCG had no effect on *Gata4* or *Gata6* mRNA levels. However, as expected, hCG and
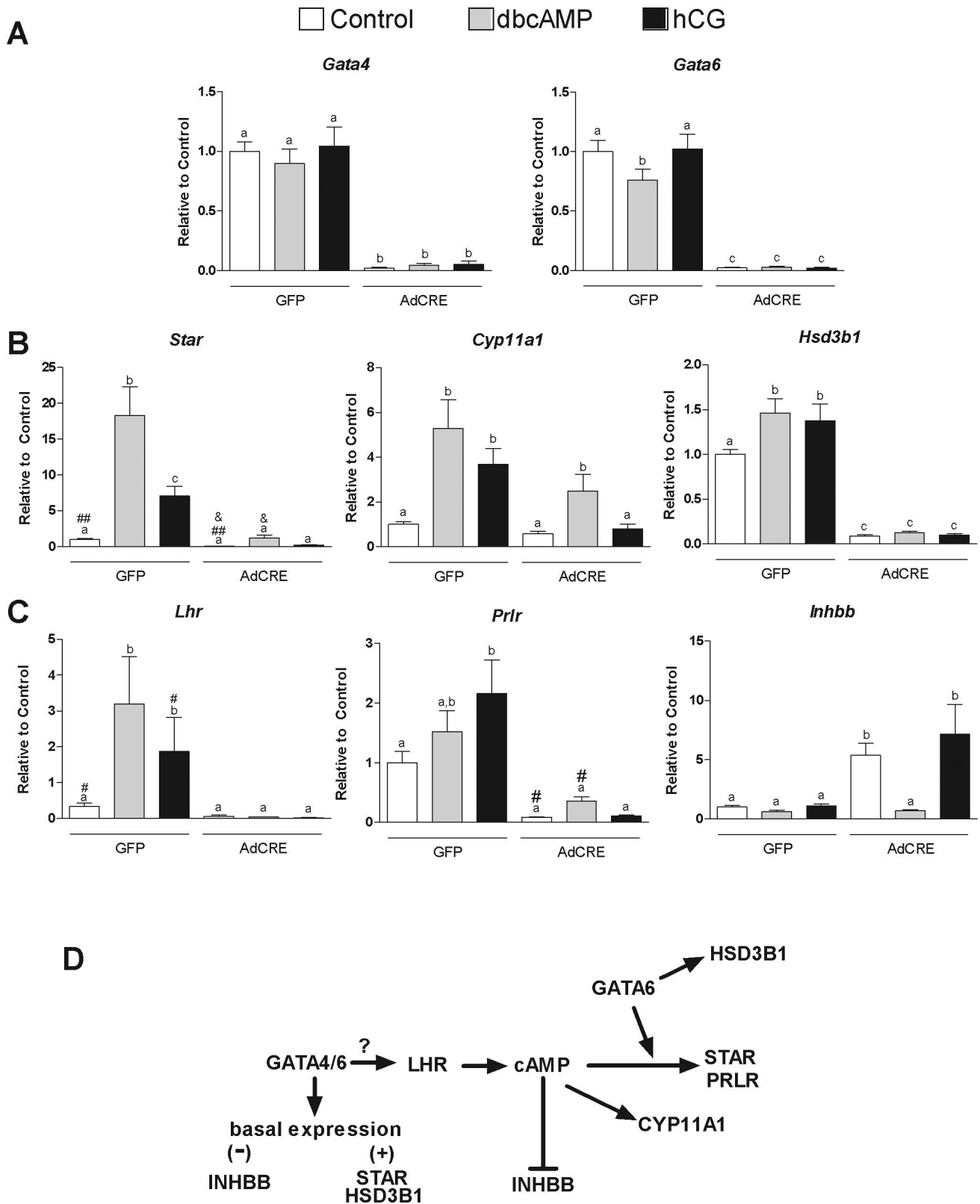
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FIG. 6. Role of LHR on the regulation of the steady-state levels of mRNA by GATA factors. **A**, **B**, and **C**) Control or *Gata4/6^{f/f}* mice were treated with 7.5 IU of eCG for 48 h followed by treatment with 7.5 IU of hCG for 7 h to induce luteinization. Luteal cells were cultured for 48 h with a Cre recombinase expression adenovirus (AdCRE) to induce recombination or a GFP expression vector as a control at a multiplicity of infection of 10. Cells were then treated for 48 h with vehicle, dibutyryl cAMP (dbcAMP) (1 mM), or hCG (50 ng/ml). Quantification of the expression of *Gata4, Gata6, Star, Cyp11a1, Hsd3b1, Lhr, Prlr,* and *Inhbb* was performed using quantitative PCR. Columns represent the mean ± SEM of four different samples. Columns with different letters differ significantly ($P < 0.05$; one-way ANOVA, Tukey test, n = 3). Paired columns labeled with #, ##, or & differ significantly from each other by *t*-test ($P < 0.05$, n = 3). **D**) General scheme indicating genes regulated directly by GATA factors or indirectly via the inhibition of LHR expression. The question mark (?) denotes possible direct or indirect effects of GATA4/6 on LHR expression.

dbcAMP treatment increased the steady-state levels of *Star, Cyp11a1,* and *Hsd3b1* mRNA levels (Fig. 6B).

In untreated GATA4 and GATA6-deficient cells, the mRNA levels of *Star* and *Hsd3b1* were significantly reduced when compared to the control cells. Moreover, S*tar, Cyp11a1,* and *Hsd3b1* mRNA levels were not stimulated by hCG in the GATA4/6-deficient cells. In marked contrast, in cells lacking GATA factors, treatment with dbcAMP caused a 16-fold increase in *Star* mRNA levels, which is similar in magnitude to the 18-fold increase induced by dbcAMP in control cells. However, dbcAMP-stimulated *Star* mRNA levels in the control cells were 18 times higher than in GATA4/6-deficient cells. Similarly, dbcAMP stimulated *Cyp11a1* to nearly the same extent in the control cells (5.2-fold) and the GATA4/6-deficient cells (4.25-fold), although in the absence of GATA4 and GATA6, *Cyp11a1* mRNA levels were approximately 50% lower when compared to the control cells. Treatment with dbcAMP had no effect on *Hsd3b1* mRNA expression in the GATA4/6-deficient cells.

In the control cells, dbcAMP and hCG significantly increased *Lhr* mRNA levels, whereas *Prlr* mRNA levels showed only a tendency to increase in dbcAMP-treated cells, although *Prlr* was significantly stimulated by hCG (Fig. 6C). In GATA4/6-deficient cells, hCG was unable to increase *Lhr* or *Prlr* mRNA expression, whereas dbcAMP increased *Prlr* mRNA but had no effect on *Lhr* mRNA levels. The increase in *Prlr* mRNA levels by dbcAMP was significant by *t*-test when compared to the controls.

Finally, we examined the mRNA levels of inhibin βA (*Inhba*) and inhibin βB (*Inhbb*), the expression of which decreases during corpus luteum formation [25, 26]. No changes in the steady-state mRNA levels of *Inhba* were observed regardless of cell genotype or treatment (data not shown). On the other hand, in untreated GATA4/6-deficient cells, the mRNA levels of *Inhbb* were significantly higher when compared to the control cells (Fig. 6C). Interestingly, *Inhbb* expression decreased in GATA4/6-deficient cells treated with dbcAMP but not in cells treated with hCG, suggesting that GATA4 and GATA6 are not required for the downregulation of *Inhbb* induced by cAMP.

DISCUSSION

This report demonstrates that GATA4 and GATA6 are crucial for normal luteal function and consequently for female fertility. Previously, we reported that GATA4 and GATA6 are required for follicle growth beyond the early antral stage [19, 20]. Here, the *Gata4* and *Gata6* genes were targeted during the luteinization process after follicle maturation. In the *PRcre:Gata4/6^{f/f}* mice, ovulation was not impaired, indicating normal follicular function; however, these mice were infertile. Our results show that in experimental animals, luteal steroidogenesis is significantly reduced within 48 h of the onset of luteinization and implantation could be rescued by treatment with P4. These findings suggest that diminished luteal P4 synthesis in *PRcre:Gata4/6^{f/f}* mice is, at least in part, responsible for the infertility observed in these animals.

The transient expression of the progesterone receptor is tightly coupled with the preovulatory LH surge and localizes exclusively to the granulosa cells, but it is absent from the theca cells of ovulating follicles [27]. It is therefore expected that in our experimental approach, the knockdown of GATA4 and GATA6 takes place only in granulosa cells. As theca cells form part of the corpus luteum in mice, the decrease in serum P4 and the infertility of the *PRcre:Gata4/6^{f/f}* mice suggest that the luteal cells derived from the granulosa cell layer are the

main contributors to circulating P4. However, because luteinizing granulosa cells may produce factors necessary for the differentiation of theca cells, we cannot rule out defects in theca cell luteinization in *PRcre:Gata4/6^{f/f}* mice.

We demonstrated that *PRcre:Gata4/6^{f/f}* mice are infertile; however, in the *PRcre:Gata4^{f/f}* and *PRcre:Gata6^{f/f}* animals, only a significant decrease in the number of pups per litter was observed. This finding suggests that GATA4 and GATA6 partially compensate for each other. In follicles, the lack of GATA6 can be fully compensated for by GATA4 [19, 20]. In sharp contrast, no full compensation takes place in the corpus luteum, as indicated by the subfertility of the *PRcre:Gata6^{f/f}* mice. A previous in vitro study suggested a more prominent role of GATA6 on luteal cell function in pigs [18]. This notion is further supported by the increase in the *Gata6* mRNA levels that we observed in the control mice after hCG treatment, which was accompanied by a simultaneous decrease in GATA4. Moreover, previous reports demonstrated that during the postovulatory luteinization period, GATA4 levels decline and remain low in the corpus luteum [1–3, 6, 28], whereas GATA6 expression is fairly strong during the postovulatory period [2, 3, 28]. In good agreement with this expression pattern, our in vivo time-course experiment indicates that the decrease in *Star, Hsd3b1,* and *Lhr* expression and in serum P4 levels correlates more closely with the reduction of *Gata6* expression than with *Gata4* mRNA levels. However, findings from the *PRcre:Gata4^{f/f}* and *PRcre:Gata6^{f/f}* mice indicate that both factors are equally required for the luteal expression of *Star, Hsd3b1, Cyp11a1,* and *Lhr* and for maintaining serum P4 levels. Therefore, although GATA6 seems to be more important, the GATA6-targeted genes required to maintain luteal cell function remain to be revealed.

Recent reports have demonstrated an essential role of GATA4 in the regulation of steroidogenesis in testicular cells. For instance, Schrade et al. [29] demonstrated that siRNA-mediated silencing of GATA4 in a murine Leydig tumor cell line downregulates the genes needed for androgen biosynthesis, including *Star, Cyp11a1, Hsd3b1,* and *Cyp17a1*. Similar findings were found with GATA4 knockdown in primary cultures of Leydig cells using the Cre/lox system. In good agreement with our findings, that report also showed a decrease in the expression of LHR in the presence of siRNA against GATA4. Interestingly, GATA4 silencing also impairs glycolysis in Leydig cells [29], and it remains possible that this pathway is targeted in luteal cells. In addition, Bergeron et al. [30] recently reported that GATA4 knockdown in mouse MA-10 Leydig tumor cells was associated with reduced expression of *Star, Cyp11a1,* and *Hsd3b1*. These previous in vitro findings and our current in vivo and in vitro results support the notion that GATA4 is a key transcriptional regulator of steroidogenesis in testicular and ovarian cells.

Our in vivo and in vitro findings, however, revealed that *Star* and *Hsd3b1* are highly sensitive to GATA6 depletion. For instance, in vivo time-course experiments demonstrated that *Star* and *Hsd3b1* mRNAs were significantly decreased as early as 48 h after hCG treatment, at the time when a significant decrease in *Gata6* expression was observed. Strikingly, we observed a 50% reduction in *Star* mRNA levels 36 h after hCG, at the time when *Gata6* was only slightly lower than in the controls. Moreover, in vitro, the basal expression of *Star* and *Hsd3b1* was reduced to almost undetectable levels in cells that expressed minimal levels of GATA. In contrast, the mRNA levels of *Cyp11a1* were significantly lower compared to the controls only 96 h after

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hCG treatment, at the time when the expression of GATA4 and GATA6 becomes significantly lower in conditional knockout animals. Taken together, these findings suggest that GATA6 is essential for the normal expression of *Star* and *Hsd3b1* in the corpus luteum.

Our findings also suggest that the mechanisms involved in the regulation of luteal *Star, Cyp11a1,* and *Hsd3b1* expression may be different for each gene. For instance, the *Star* and *Hsd3b1* genes are directly regulated by GATA6, whereas both GATA4 and GATA6 seem to indirectly regulate *Cyp11a1*. In fact, a novel finding of this report is an early block on *Lhr* expression in luteinizing granulosa cells of *PRcre:Gata4/6^{f/f}* mice. Therefore, we evaluated to what extent diminished LHR signaling contributes to the block in steroidogenesis. The findings suggested the presence of three groups of genes based on whether or not their stimulation by cAMP requires GATA4/6 or whether GATA factors only enhance the effects of LH on gene expression. Thus, cAMP stimulation of *Hsd3b1* and *Lhr* requires GATA. On the other hand, cAMP is able to stimulate *Star* and *Prlr* in the absence of GATA4 and GATA6, but both GATA factors are necessary for the full stimulation of these genes. In contrast, the stimulation of *Cyp11a1* by cAMP does not require GATA (Fig. 6D). The findings suggest that in mice the downregulation of *Lhr* leads to a decrease in *Cyp11a1* expression and to a diminished induction of *Star* and *Hsd3b1* by hCG.

LHR is crucial for luteal formation and function [31, 32]. It is well known that after the LH surge, *Lhr* mRNA and protein levels drop due to a decrease in *Lhr* mRNA half-life [33–37]. However, this drop lasts for approximately 36 h, and the receptor becomes highly expressed in luteal cells [22, 34, 35, 38]. Our findings demonstrate that in luteal cells expressing low levels of GATA4 and GATA6, *Lhr* mRNA drops to almost undetectable levels, suggesting that GATA factors play a key role in the upregulation of LHR in the corpus luteum. It remains to be determined whether GATA inhibits the mechanisms involved in *Lhr* mRNA degradation or directly stimulates the activity of the *Lhr* promoter in luteal cells (question mark in Fig. 6D).

Our findings also indicate that silencing of the *Inhbb* gene in luteal cells does not require GATA factors. *Inhbb* is highly expressed in preovulatory granulosa cells and is downregulated during luteinization by the action of LH [25, 26]. Interestingly, we observed that knockdown of GATA4 and GATA6 increases the basal expression of *Inhbb,* whereas treatment with cAMP, but not hCG, decreases the elevated levels of *Inhbb* mRNA found in luteal cells lacking GATA4 and GATA6. These results suggest that GATA stimulation of LHR is required for the downregulation of *Inhbb* that takes place during luteinization. Of great interest, Bergeron et al. [30] recently demonstrated that *Inha* and *Inhba* are decreased by the knockdown of GATA4 in mouse MA-10 Leydig tumor cells, whereas we found no effect of GATA4/6 knockdown on *Inhba*. These findings suggest that the effect of GATA on the inhibin system are cell specific.

The main function of the corpus luteum is to synthesize high levels of P4, which is critical for the maintenance of pregnancy. To evaluate whether the loss in P4 synthesis is the primary cause of infertility in *PRcre:Gata4/6^{f/f}* mice, we attempted to rescue pregnancy in these animals by administrating P4. Progesterone replacement rescued implantation in *PRcre:Gata4/6^{f/f}* females, although it was not effective in every animal. However, none of the *PRcre:Gata4/6^{f/f}* animals that received sesame oil showed signs of implantation. These findings suggest that luteal dysfunction is one of the main causes of infertility in *PRcre:Gata4/6^{f/f}* mice. Nevertheless, we cannot discard defects in the oviducts and/or uterus. In fact, despite the fact that the knockdown of either GATA4 or GATA6 equally affects steroidogenesis, only *PRcre:Gata4/6^{f/f}* mice are infertile. Therefore, the ultimate cause of infertility remains elusive, and further experiments are needed to determine whether GATA4/6 regulate oviductal and uterine function.

In summary, our findings demonstrate that GATA4 and GATA6 are essential for luteal P4 synthesis in mice. The knockdown of GATA4 and GATA6 severely reduces the response of luteal cells to gonadotropins. Although the mechanisms by which GATA4 and GATA6 control *Lhr* and *Prlr* expression remain to be elucidated, our findings provide novel in vivo evidence indicating that GATA factors are crucial regulators of luteal steroidogenesis and gonadotropin receptor expression.

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